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SIRT6 regulates the cigarette smoke-induced signalling in rheumatoid arthritis synovial fibroblasts

Engler, Anna ; Niederer, Fabienne ; Klein, Kerstin ; Gay, Renate E ; Kyburz, Diego ; Camici, Giovanni G ; Gay, Steffen ; Ospelt, Caroline

Abstract: Cigarette smoking is a recognized environmental risk factor for the development and progression of rheumatoid arthritis (RA). RA synovial fibroblasts (RASf) actively contribute to inflammation and joint destruction in this chronic inflammatory autoimmune disease. In the current study, we investigated the influence of cigarette smoke on the inflammatory and matrix-destructive properties of RASf. Furthermore, the functional role of Sirtuin 6 (SIRT6) in the regulation of the signalling induced by cigarette smoke or by tumor necrosis factor alpha (TNF) was elucidated. We demonstrated that stimulation with cigarette smoke extract (CSE) enhances the pro-inflammatory and matrix-destructive potential of RASf by inducing the production of pro-inflammatory cytokine interleukin 8 (IL8) and the matrix-destructive enzyme matrix metalloproteinase 1 (MMP1), but not of IL6 and MMP3. Moreover, we could show that the expression of MMP1 is specifically regulated by SIRT6. Treatment of RASf with CSE or TNF increased the levels of SIRT6. The expression of SIRT6 was also enhanced in vivo in synovial tissues of RA smokers and in joints of mice exposed to cigarette smoke. Silencing of SIRT6 specifically increased basal as well as CSE- and TNF-induced production of MMP1, demonstrating that SIRT6 plays an important role in restricting MMP1 expression. In conclusion, the upregulation of SIRT6 in RASf under CSE or TNF stimulation functions as a counterregulatory mechanism attenuating the production of the matrix-destructive enzyme MMP1. This is the first study revealing the protective function of SIRT6 in the cigarette smoke-induced signalling. **KEY MESSAGES:** Cigarette smoke induces pro-inflammatory and matrix-destructive responses in RASf. Cigarette smoke enhances the expression of SIRT6 in vitro and in vivo. TNF increases the levels of SIRT6. SIRT6 diminishes MMP1 production under cigarette smoke extract and TNF stimulation.

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SIRT6 regulates the cigarette smoke-induced signalling in rheumatoid arthritis synovial fibroblasts

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Abstract

Cigarette smoking is a recognized environmental risk factor for the development and progression of rheumatoid arthritis (RA). RA synovial fibroblasts (RASf) actively contribute to inflammation and joint destruction in this chronic inflammatory autoimmune disease. In the current study, we investigated the influence of cigarette smoke on the inflammatory and matrix-destructive properties of RASf. Furthermore, the functional role of Sirtuin 6 (SIRT6) in the regulation of the signalling induced by cigarette smoke or by tumor necrosis factor alpha (TNF α) was elucidated. We demonstrated that stimulation with cigarette smoke extract (CSE) enhances the pro-inflammatory and matrix-destructive potential of RASf by inducing the production of pro-inflammatory cytokine interleukin 8 (IL8) and the matrix-destructive enzyme matrix metalloproteinase 1 (MMP1), but not of IL6 and MMP3. Moreover, we could show that the expression of MMP1 is specifically regulated by SIRT6. Treatment of RASf with CSE or TNF α increased the levels of SIRT6. The expression of SIRT6 was also enhanced in vivo in synovial tissues of RA smokers

and in joints of mice exposed to cigarette smoke. Silencing of SIRT6 specifically increased basal as well as CSE- and TNF α -induced production of MMP1, demonstrating that SIRT6 plays an important role in restricting MMP1 expression. In conclusion, the upregulation of SIRT6 in RASf under CSE or TNF α stimulation functions as a counterregulatory mechanism attenuating the production of the matrix-destructive enzyme MMP1. This is the first study revealing the protective function of SIRT6 in the cigarette smoke-induced signalling.

Key messages

- Cigarette smoke induces pro-inflammatory and matrix-destructive responses in RASf.
- Cigarette smoke enhances the expression of SIRT6 in vitro and in vivo.
- TNF α increases the levels of SIRT6.
- SIRT6 diminishes MMP1 production under cigarette smoke extract and TNF α stimulation.

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Matrix metalloproteinases · Cytokines

Introduction

Rheumatoid arthritis (RA) is an inflammatory disorder affecting multiple joints and leading to progressive destruction of cartilage and bone. Along with inflammatory cells, RA synovial fibroblasts (RASf) actively contribute to inflammation and joint destruction [1]. RASf exhibit an activated and aggressive phenotype, characterized by increased resistance to apoptosis, enhanced production of inflammatory cytokines, matrix-degrading enzymes, and pro-angiogenic factors [2].

Multiple mechanisms including genetic predisposition, epigenetic changes, interaction with matrix components, and inflammatory mediators contribute to the activation of RASF [3]. The impact of environmental factors on the activated phenotype of RASF is unknown. Smoking is a recognized environmental risk factor not only for the development of RA, but also for the severity of established RA. Specifically, smoking was associated with increased disease activity and progression [4–6]. RA patients with a history of smoking were reported to show poor response to anti-tumor necrosis factor (TNF) therapy and RA heavy smokers, to have the poorest drug survival [7, 8]. The role of cigarette smoke in the development of collagen-induced arthritis (CIA) in mice is less clear. Lindblad and colleagues have shown that cigarette smoke exposure delays the onset and progression of CIA in mice [9]. However, in two other studies the treatment of mice with cigarette smoke condensate extract augmented the development of CIA [10, 11].

Since synovial fluid is a passive ultrafiltrate from the blood, substances in the blood stream, e.g., drugs and their metabolites, can be found or even accumulate in the synovial fluid and possibly alter joint physiology [12, 13]. Indeed, gene expression in synovia from smoking RA patients was significantly altered compared to nonsmoking RA patients [14]. Furthermore, components of cigarette smoke were shown to increase invasion of RASF in Matrigel in vitro [15]. However, the specific regulators and the signalling pathways that are affected by cigarette smoke in RA are poorly investigated. In particular, it is not clear how cigarette smoke regulates the gene expression that influences the activity of RASF.

Sirtuins (SIRT6) regulate a broad range of cellular processes including aging, metabolism, cell survival, gene transcription, stress responses, and inflammation [16]. In mammals, seven members of the sirtuin family (SIRT1–SIRT7) were identified that regulate the activity of histones, transcription factors, and other target proteins by posttranslational modifications [17, 18]. We have previously reported that SIRT1 overexpression in RA synovium contributes to pro-inflammatory cytokine production and apoptosis resistance [19]. Another member of the sirtuin family, SIRT6, has gained much attention in recent years because of its role in DNA repair and genomic stability as well as in suppression of tumor formation and NF- κ B signalling [20]. In a recent publication by Lee et al., the overexpression of SIRT6 was shown to inhibit the expression of NF- κ B target genes and to decrease the severity of CIA in mice, demonstrating an important protective function of SIRT6 [21].

In the current study, we investigated the influence of the environmental risk factor of cigarette smoke on the gene expression that regulates the inflammatory and matrix-destructive properties of RASF. We demonstrated that an

increase in SIRT6 levels is important for restriction of cigarette smoke- and TNF α -induced production of the matrix-destructive enzyme MMP1.

Methods

Patient samples/cell culture

Synovial tissues were obtained from RA patients undergoing joint replacement surgery at the Schulthess Clinic Zurich after written informed consent according to the principles of the 1964 Declaration of Helsinki. For all experiments with human and mouse tissues, ethical approval was obtained at the Swiss Ethical commission. All RA patients fulfilled the American College of Rheumatology criteria for the classification of RA [22]. For fibroblast cultures, tissue specimen were digested with collagenase and cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % heat inactivated fetal calf serum, 50 IU/ml penicillin-streptomycin, 2 mM l-glutamine, 10 mM HEPES, and 0.2 % fungicide. For experiments, RASF from passages 4 to 8 were used. The characteristics of patients used for detection of SIRT6 in synovial tissues are shown in Table 1.

Animals

Male C57BL/6 mice ($n=6$) were exposed to cigarette smoke from research cigarettes (University of Kentucky 1R4F) twice a day for a total of 6 h, 5 days per week in a whole-body smoking chamber (Teague TE-10). Cigarettes were smoked at a rate of a 2 s, 35 ml puff each minute and smoke particulate concentration was kept at 25.2 mg/m³. Control mice ($n=8$) were exposed to filtered ambient air. After 3 weeks, mice were euthanized, and mRNA from dissected joints was isolated.

Stimulation assays

Cigarette smoke extract (CSE) was prepared using a modification of the method described by Vassallo et al. [23]. One-hundred-percent CSE was prepared by bubbling the smoke of one cigarette into 10 ml of cell culture medium at a rate of one cigarette per 7 min. The generated CSE was sterile-filtered and diluted to 5 % CSE with cell culture medium. The pH of 5 % CSE and of the control cell culture medium was equal and remained unaltered during the cultivation. CSE preparations were freshly prepared for each experiment and monitored by measuring the optical density at 320 and 350 nm. The cigarettes used contained 10 mg tar, 0.8 mg nicotine, and 10 mg carbon monoxide (Marlboro

Table 1 Patient characteristics used for detection of SIRT6 in synovial tissues

Characteristics	RA smoker (<i>n</i> =11)	RA non smoker (<i>n</i> =11)
Age; years, mean (range)	60 (53–73)	64 (40–78)
Sex; female/male	7/4	10/1
Number of cigarettes per day; mean (range)	13 (2–20)	–
Disease duration; years, mean (range)	17 (4–28) (2 patients ND)	25 (4–50)
CRP; mg/l, mean (range)	9 (0.1–19.3) (4 patients ND)	6 (0.1–20.2) (6 patients ND)
RF positive	9/10 (1 patient ND)	5/5 (6 patients ND)
Medications		
DMARDs	5	8
NSAIDs	4	2
steroids	4	1
biologics	1	4
none	1	2

Age, disease duration, and CRP were not significantly different between RA smokers and RA nonsmokers

ND not determined, NSAIDs non-steroidal anti-inflammatory drugs, DMARDs disease-modifying anti-rheumatic drugs, RF rheumatoid factor, CRP C-reactive protein

Red). Confluent cells were stimulated with 5 % CSE or 10 ng/ml TNF α for 24 h.

Transfection experiments

RASF were transfected using the Basic Nucleofector Kit for Primary Mammalian Fibroblasts (Amaxa/Lonza). For SIRT6 silencing, 10 μ M SIRT6-validated small interfering RNA (siRNA) or control siRNA (both Qiagen) were used. Transfected RASF were cultured for 48 h and subsequently stimulated with CSE or TNF α .

RNA isolation and quantitative real time PCR

Total RNA was isolated using the RNeasy Miniprep Kit (Qiagen) including DNase treatment. RNA was dissolved in RNase-free water, and the optical density was determined at 260 nm in a spectrophotometer. RNA was reverse transcribed using random hexamers and multiscribe reverse transcriptase (Applied Biosystems). Samples without addition of reverse transcriptase were used as negative controls (non-RT). Relative quantification of mRNA levels was performed by SYBRGreen Real time PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) for 40 cycles. As endogenous controls, hypoxanthine phosphoribosyltransferase 1 (HPRT1) was used for human samples and 18 s rRNA assay for murine samples (Life Tech-Applied Biosystems). The differences of the comparative threshold cycles (Ct) of sample and endogenous control were calculated (dCt). Relative expression levels were calculated following the formula $\text{ddCt} = \text{dCt} (\text{sample stimulated}) - \text{dCt} (\text{sample unstimulated})$, relative expression was calculated using the expression $2^{-\text{ddCt}}$. For primer sequences see supplementary table S1.

Enzyme linked immunosorbent assay (ELISA)

Proteins in cell supernatants were detected with OptEIA[®] Kits (BD Pharmingen) for IL6, IL8, and TNF α , and with DuoSet ELISA Development Kits (R & D Systems) for MMP1 and MMP3. Data were analyzed using Revelation v4.22 software (Dynex Technologies).

Immunohistochemistry

Formalin-fixed, paraffin-embedded synovial tissue sections were deparaffinised, pretreated with EDTA (pH 9), blocked for endogenous peroxidase activity (3% H₂O₂) and for non-specific binding (5 % goat serum/1 % bovine serum albumin), and incubated with rabbit IgG (Jackson ImmunoResearch) or rabbit anti-SIRT6 antibodies (D8D12, Cell Signaling, #12486). SIRT6-positive cells were visualized with biotinylated goat anti-rabbit antibodies (Jackson ImmunoResearch) and DAB (3,3'-Diaminobenzidin, Vector Laboratories). For double stainings, the slides were stained with anti-SIRT6 as described above. Following, the slides were pretreated with 10 mM citrate buffer (pH 6) or 1 mg/ml trypsin, blocked with 5 % goat serum/1 % bovine serum albumin, and incubated with mouse anti-prolyl 4-hydroxylase beta (Acris Antibodies) or with mouse anti-CD68 (Dako). Slides were incubated with biotinylated goat anti-mouse antibodies (Jackson ImmunoResearch). Prolyl 4-hydroxylase beta-positive cells were visualized with HistoGreen (Linaris) and CD-68-positive cells with Vector Blue Alkaline Phosphatase Substrate Kit (Vector Laboratories). Slides stained with single anti-SIRT6 or IgG control were counterstained with hematoxylin. The images were obtained on a Zeiss Axio Imager Z1 microscope through a Zeiss Plan-Neofluar 10 \times /0.30 or 40 \times /0.75 numerical objective aperture and acquired by a Zeiss AxioCam HRC camera and the AxioVision 4.6.3.SP1 imaging

software (Carl Zeiss). The intensity of SIRT6 staining was evaluated by two blinded observers, using a gradual scoring scale from 0 (no staining) to 4 (strong staining).

Immunoblotting

Whole cell lysates were prepared for immunoblotting as previously described [19]. Nuclear fractions were enriched by differential centrifugation at 4 °C. RASF were washed in PBS and harvested by cell scraping. Following centrifugation, the cell pellet was incubated in homogenization medium (150 mM MgCl₂, 10 mM KCl, 10 mM TrisHCl (pH 6.7)) for 5 min. The cell suspension was homogenized using a glass-glass homogenisator and centrifuged at 1,000g for 10 min. The pellet was lysed in RIPA buffer. The following antibodies were used: anti-SIRT4 (Abcam), anti-SIRT6 (Cell Signaling, #2590), anti-Vimentin (Abcam), or anti-histone H3 (Abcam). Horseradish peroxidase (HRP)-labeled species-specific secondary antibodies (Jackson ImmunoResearch) and enhanced chemiluminescence (GE Healthcare) were used for visualization.

Statistical analysis

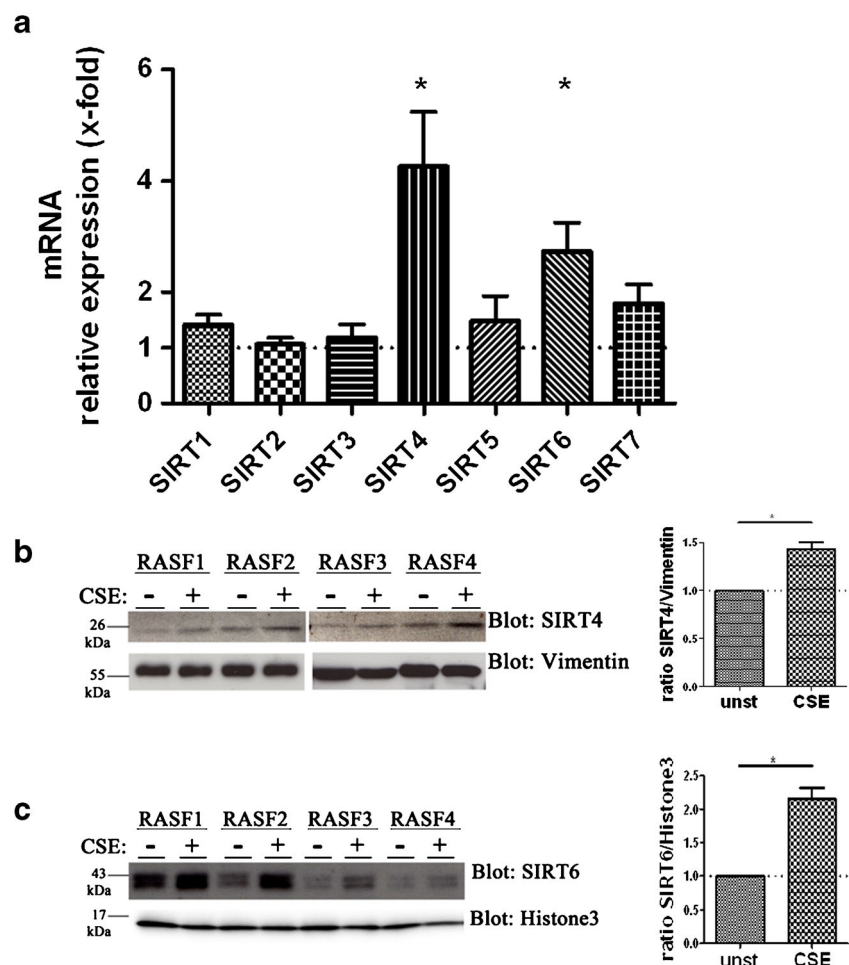
Statistical analysis was performed using GraphPad Prism 5.0 software. Values are presented as mean±standard error of mean (SEM). After testing the values for Gaussian distribution, data were analyzed using one sample *t* test, Mann–Whitney, or Wilcoxon matched-pairs signed rank test as indicated. Two-way analysis of variance (ANOVA) was used to determine synergistic or additive effects. *p* values <0.05 were considered as significant.

Results

Cigarette smoke alters the expression of sirtuins in vitro and in vivo

To investigate the impact of cigarette smoke on the expression of sirtuins, RASF from nonsmokers were stimulated with 5 % CSE for 24 h. Among all sirtuins examined (SIRT1–7), the transcriptional level of SIRT4 was 4.3-fold (*p*=0.01) and

Fig. 1 Stimulation of RASF with cigarette smoke extract (CSE) increases the expression of sirtuins. **a** Confluent monolayer cultures of synovial fibroblasts from patients with RA (*n*=6) were treated with 5 % CSE for 24 h. Expression of *SIRT1–7* mRNA was measured by real time PCR using specific primers and normalized to the housekeeping gene *HPRT1*. Changes in the transcriptional levels of *SIRTs* are shown as fold induction compared to untreated cells (dotted line). **(b–c)** Protein expression of *SIRT4* and *SIRT6* was increased in synovial fibroblasts from patients with RA (=RASF, *n*=4) stimulated with CSE. Molecular weights are indicated in kiloDalton (kDa). Bars show the mean±SEM. *n*=number of patients investigated. **p*<0.02, by one sample *t* test



SIRT6 mRNA was 2.7-fold ($p=0.02$) enhanced in CSE-stimulated RASF (Fig. 1a). Elevated expression of SIRT4 and SIRT6 was confirmed at the protein level by immunoblot (Figs. 1b, c).

Increased mRNA expression of SIRT4 and SIRT6 was detected in vivo in joints of mice exposed to cigarette smoke for 3 weeks. The basal mRNA levels of SIRT4 were 1.8-fold higher (Δ CT smoking 14.6 ± 0.6 ; Δ CT control 15.5 ± 0.7 ; $p=0.04$) and of SIRT6, 2.3-fold higher (Δ CT smoking 13.6 ± 0.9 ; Δ CT control 14.8 ± 1.0 ; $p=0.04$) in joints of mice exposed to cigarette smoke as compared to control mice (Fig. 2a). In human synovial tissue samples from RA patients, smokers had 1.6-fold higher mRNA expression of SIRT6 (Δ CT smokers 5.8 ± 0.7 ; Δ CT nonsmokers 6.5 ± 0.6 ; $p=0.04$) as compared to nonsmokers (Fig. 2b). The levels of SIRT4 mRNA were not significantly different in synovial tissues of RA patients who smoked.

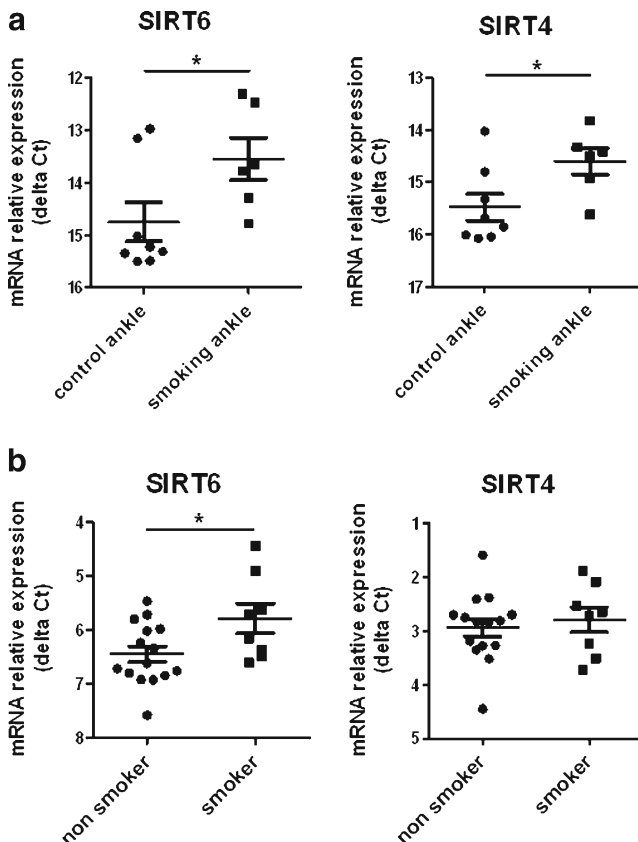


Fig. 2 Exposure to cigarette smoke enhances the expression of sirtuins in vivo. **a** Increased mRNA levels of SIRT6 and SIRT4 in the ankles of mice exposed to cigarette smoke (*smoking ankle*, $n=6$) as compared to control treated mice (*control ankle*, $n=8$). Changes in the transcriptional levels of SIRT4 and SIRT6 are shown as the difference in threshold cycle (Δ Ct), relative to 18S rRNA. **b** Elevated expression of SIRT6 transcript in human synovial tissue samples from RA patients who smoked (*smoker*) as compared to RA nonsmokers (*nonsmoker*). Relative expression of mRNA is presented as the difference in threshold cycle (Δ Ct), relative to HPRT1. Bars show the mean \pm SEM. * $p < 0.04$, by Mann–Whitney test

We further investigated the expression of SIRT6 in synovial tissue sections from RA patients by immunohistochemistry. Scoring of SIRT6 staining showed increased expression of nuclear SIRT6 in synovial tissues of RA smokers when compared to RA nonsmokers (Figs. 3a, b). To detect the specific cells in the synovium that express SIRT6, we performed double stainings with SIRT6 and prolyl 4-hydroxylase beta as a fibroblast marker or CD68 as a macrophage marker. Strong expression of SIRT6 was detected in prolyl 4-hydroxylase beta-positive SF and in CD68-positive macrophages (Fig. 3c). Interestingly, the levels of SIRT6 positively correlated with the disease duration in RA smokers, but not in RA nonsmokers (Fig. 3d).

CSE increases the expression of IL8 and MMP1 in RASF

The activated and aggressive phenotype of RASF is characterized by increased production of pro-inflammatory ILs and matrix-destructive MMPs [1, 2]. In particular, $\text{TNF}\alpha$ strongly induces the secretion of ILs and MMPs in RASF. To investigate whether inflammatory and matrix-destructive properties of RASF can be modulated by CSE alone and together with $\text{TNF}\alpha$, RASF were stimulated with CSE, $\text{TNF}\alpha$, or with $\text{TNF}\alpha$ and CSE in combination. RASF released 2.1-fold more MMP1 and 1.5-fold more IL8 protein (both $p < 0.03$) in cell culture supernatants after stimulation with CSE (Fig. 4a). Secretion of IL6 and MMP3 was not altered. Co-treatment with CSE and $\text{TNF}\alpha$ led to a further increase in protein release of IL8 and MMP1 compared to stimulation of RASF with $\text{TNF}\alpha$ alone (Fig. 4a), but did not affect $\text{TNF}\alpha$ -mediated secretion of IL6 or MMP3.

Real time PCR analysis confirmed 6.5-fold enhanced levels of MMP1 transcript and 3.5-fold increased expression of IL8 mRNA after CSE treatment (both $p < 0.02$) (Fig. 4b), indicating that CSE induces changes at the transcriptional level. Stimulation of RASF with CSE and $\text{TNF}\alpha$ in combination further increased mRNA levels of MMP1 and IL8 compared to the stimulation with $\text{TNF}\alpha$ alone (Fig. 4b). Two-way analysis of variance (ANOVA) revealed that IL8 production was synergistically induced by CSE and $\text{TNF}\alpha$ whereas an additive effect of CSE and $\text{TNF}\alpha$ was detected on MMP1 expression. Moreover, mRNA levels of $\text{TNF}\alpha$ and IL-1 β were measured; however, they were under the detection limit before as well as after stimulation with CSE.

In summary, these results indicate that CSE increases the transcription and secretion of IL8 and MMP1, and further augments the strong effect of $\text{TNF}\alpha$ on the expression of IL8 and MMP1 thereby promoting the pro-inflammatory and matrix-destructive potential of RASF.

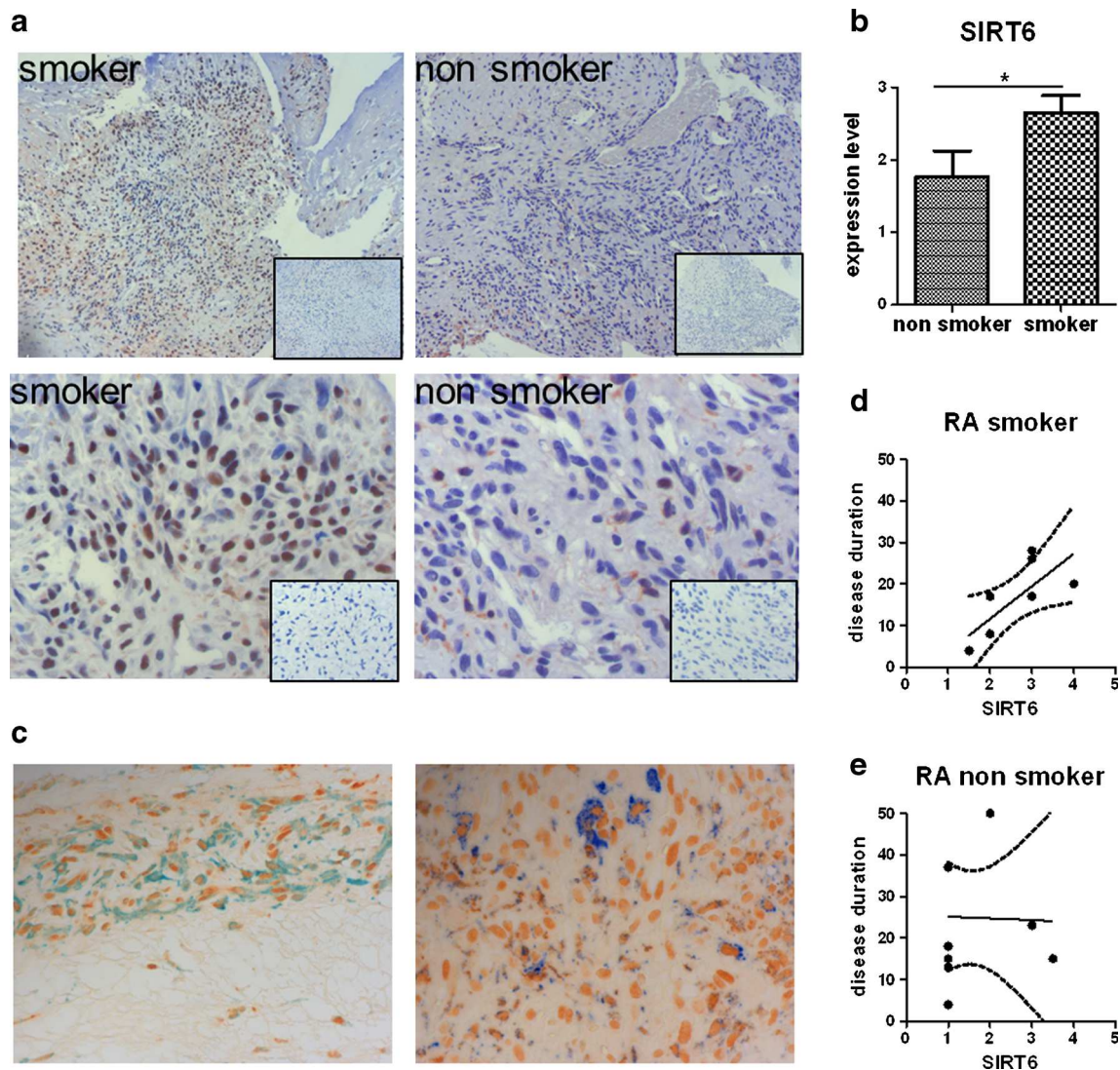


Fig. 3 Expression and localization of SIRT6 in RA synovial tissues. **a** Representative sections of RA synovial tissues stained for SIRT6 or IgG control (inset) showing increased expression of SIRT6 (brown) in RA smokers (left). Nuclei were counterstained with hematoxylin (blue). SIRT6 staining appears in brown. Original magnification: $\times 100$ (top) and $\times 400$ (bottom). **b** Staining intensities in synovial tissues of RA smokers ($n=10$) and RA nonsmokers ($n=11$) were scored. n =number

of patients investigated. Bars show the mean \pm SEM. $*p<0.04$, by Mann–Whitney test. **c** Representative sections of RA synovial tissue double stained for detection of SIRT6 (orange) and prolyl 4-hydroxylase beta (blue, left) as a fibroblast marker or CD68 (blue, right) as a macrophage marker. Original magnification: $\times 400$. Levels of SIRT6 positively correlated with the disease duration in RA smokers (**d**), but not in RA nonsmokers (**e**)

The levels of SIRT6 are increased upon stimulation with $\text{TNF}\alpha$

Since the expression of SIRT6 was altered in vitro in RASF as well as in vivo in the joints of mice and most importantly in the synovium of RA smokers, we focused on the functional role of SIRT6 in the regulation of pro-inflammatory and matrix-destructive activities of RASF. SIRT6 was shown to regulate the expression of $\text{TNF}\alpha$ -induced genes [21, 24]. However, the impact of $\text{TNF}\alpha$ on the expression of SIRT6 has not been investigated so far. To elucidate whether $\text{TNF}\alpha$ can modify the expression of SIRT6, RASF were stimulated with $\text{TNF}\alpha$. Similar to CSE, treatment with $\text{TNF}\alpha$ increased

the protein levels of SIRT6 (Fig. 5a). However, while CSE significantly enhanced the levels of SIRT6 mRNA, stimulation with $\text{TNF}\alpha$ did not affect the transcriptional levels of SIRT6 pointing to posttranscriptional regulation of SIRT6 protein in $\text{TNF}\alpha$ -treated RASF (Fig. 5b).

SIRT6 attenuates CSE- and $\text{TNF}\alpha$ -induced MMP1 expression

To investigate whether increased SIRT6 levels influence the CSE- and $\text{TNF}\alpha$ -induced production of IL8 and MMP1, upregulation of SIRT6 was inhibited with siRNA during stimulation and the secretion of ILs and MMPs was measured.

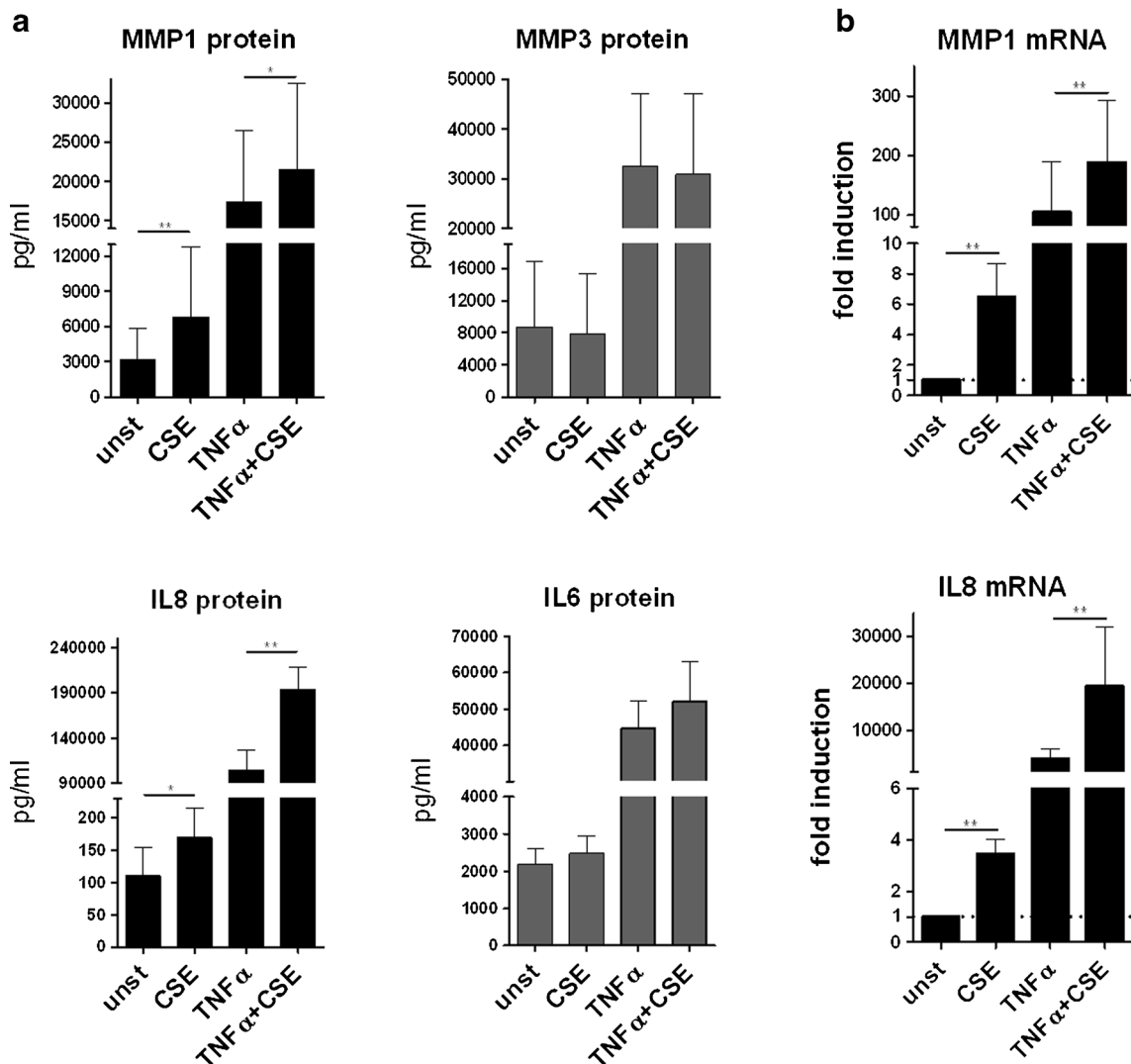


Fig. 4 Stimulation of RASF with CSE induces the expression of IL8 and MMP1. Synovial fibroblasts from patients with RA ($n=7$) were stimulated with 5 % CSE, 10 ng/ml $TNF\alpha$ or with CSE and $TNF\alpha$ in combination for 24 h. **a** Protein release of MMP1, MMP3, IL8 and IL6 was measured in cell culture supernatants of stimulated and unstimulated (unst) cells by ELISA. **b** Increased expression of MMP1 and IL8 was

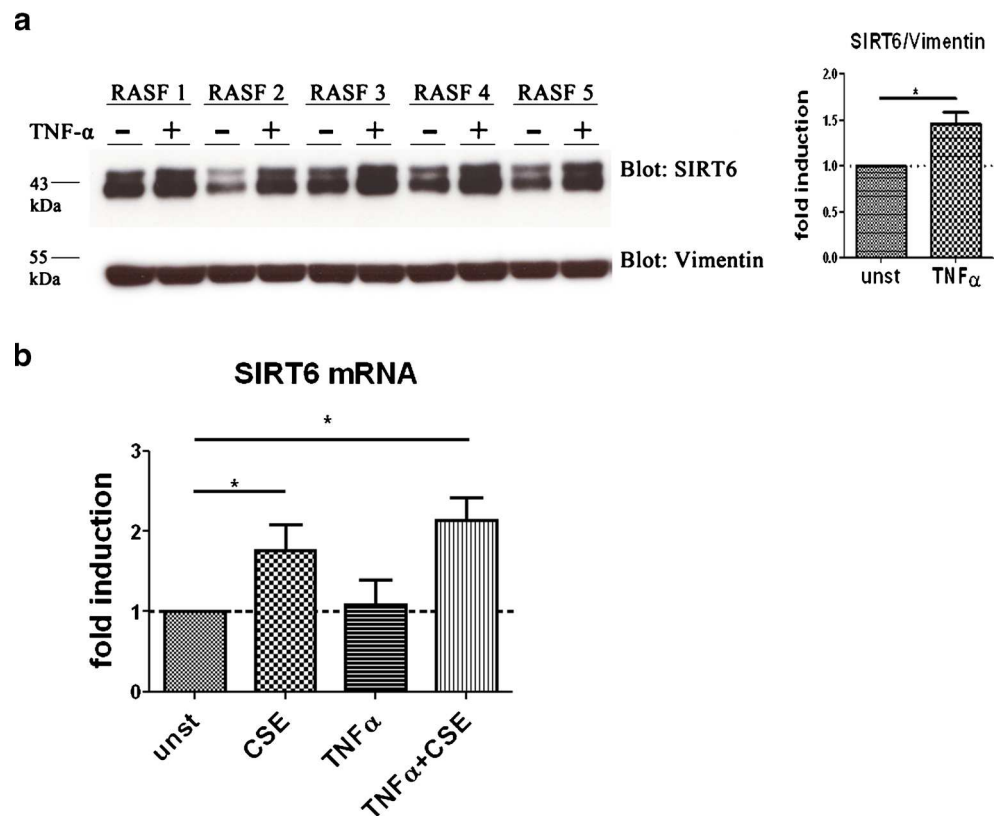
confirmed at the mRNA level in RASF stimulated with CSE using Real time PCR analysis and is shown as fold induction compared to untreated cells (unst). As endogenous control, the expression of HPRT1 mRNA was used for normalization. Values are mean \pm SEM. * $p<0.05$; ** $p<0.02$; by Wilcoxon matched-pairs signed rank test

To compare the effects of CSE and $TNF\alpha$, SIRT6-silenced cells were stimulated with CSE or $TNF\alpha$ alone or with $TNF\alpha$ and CSE in combination. Basal expression as well as induction of SIRT6 after stimulation with CSE or $TNF\alpha$ was successfully blocked by transfection of RASF with SIRT6-specific siRNA (Fig. 6a). Silencing of SIRT6 significantly increased the basal protein secretion of MMP1, but did not affect the secreted levels of MMP3, IL6, or IL8 (Fig. 6b). Additionally, under stimulation with CSE or $TNF\alpha$, the release of MMP1 was significantly enhanced in SIRT6-silenced cells as compared to control-transfected cells. Furthermore, under combined stimulation with $TNF\alpha$ and CSE, the silencing of SIRT6 led to a higher increase in MMP1 release (Fig. 6b).

To investigate whether SIRT6 can regulate the gene expression of MMP1, we measured MMP1 transcripts in SIRT6-silenced RASF. Similar to the protein levels, silencing of SIRT6 significantly increased mRNA expression of MMP1 under basal conditions as well as under stimulation with CSE or with $TNF\alpha$, or under stimulation with $TNF\alpha$ and CSE together (Fig. 6c). The expression of IL8 mRNA was not significantly altered by silencing of SIRT6.

These data show that SIRT6 reduction leads to a specific increase in the mRNA and protein expression of MMP1 under CSE as well as under $TNF\alpha$ stimulation, but does not affect the production of IL8, IL6, or MMP3. Thus, the increase in SIRT6 levels detected in

Fig. 5 $\text{TNF}\alpha$ stimulation increases the protein levels of SIRT6 in RASF. **a** Confluent monolayer cultures of synovial fibroblasts from RA patients ($=\text{RASF}$, $n=5$) were stimulated with 10 ng/ml $\text{TNF}\alpha$ for 24 h. Protein expression of SIRT6 was increased upon $\text{TNF}\alpha$ treatment. Molecular weights are indicated in kiloDalton (*kDa*). **(b)** Expression of SIRT6 mRNA was measured in synovial fibroblasts from RA patients ($n=6$) stimulated with 5 % CSE, 10 ng/ml $\text{TNF}\alpha$ or with CSE and $\text{TNF}\alpha$ together for 24 h by Real time PCR and normalized to the housekeeping gene *HPRT1*. Changes in the transcriptional levels of SIRT6 are shown as *fold induction* \pm SEM compared to unstimulated cells (*unst*). n = number of patients investigated. $*p<0.03$, by Wilcoxon matched-pairs signed rank test



CSE and $\text{TNF}\alpha$ -stimulated RASF might be a counterregulatory mechanism to restrict the production of MMP1 under cellular stress.

Discussion

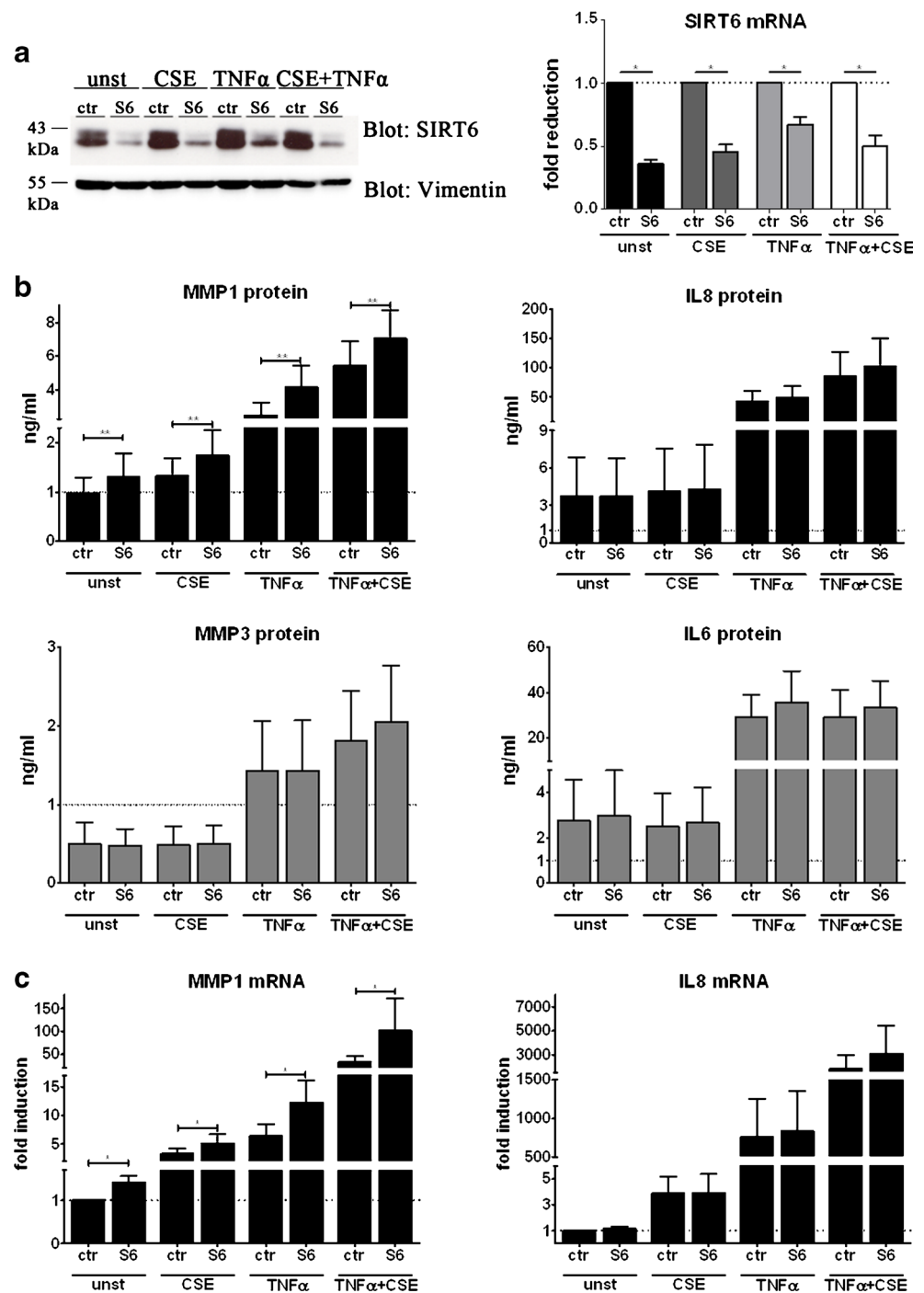
In the current study, we have investigated the molecular pathways that are altered by the environmental factor cigarette smoke in RASF. We found that stimulation with CSE enhances the pro-inflammatory and matrix-destructive properties of RASF by inducing the expression of IL8 and MMP1. Moreover, we have shown for the first time that cigarette smoke increases the expression of SIRT6 in vivo in the joints of mice and in the synovial tissues of RA patients and in vitro in RASF. Most interestingly, we found that SIRT6 specifically attenuates the transcription and secretion of the matrix-destructive enzyme MMP1 in RASF. Thus, an increase in SIRT6 levels after stimulation with CSE or $\text{TNF}\alpha$ functions as a counterregulatory mechanism to restrict MMP1 production in response to CSE- or $\text{TNF}\alpha$ -induced MMP1 expression.

Our finding of elevated levels of SIRT6 in joints of smoke-exposed mice and in synovial tissues of smokers validates our in vitro model and supports the hypothesis that cigarette smoke constituents reach the synovial fluid and cause changes

in gene expression. Currently, no study is published that analyzed the presence of cigarette smoke ingredients in the synovial fluid. In addition to our study, indirect evidence is provided by a study showing activation of aryl hydrocarbon receptors in the synovium of smokers [14]. This receptor binds polycyclic aromatic hydrocarbons, which are formed in cigarette smoke. Another study demonstrated the presence of tetrahydrocannabinol in the synovial fluid of hashish smokers, proving that bulky molecules can be filtered in the synovial fluid [25].

SIRT6 was previously shown to be induced by oxidative stress [26], and its increased expression in smokers points to the activation of stress response pathways by smoking. This is also in line with our observations that citrullination and heat shock proteins are elevated in the joints of smoke exposed mice, both of which are known to be increased under cellular stress [27]. Interestingly, cigarette smoke and $\text{TNF}\alpha$ had different effects on transcription of SIRT6. Both pro-inflammatory stimuli increased protein levels of SIRT6. However, only cigarette smoke increased the transcription of SIRT6. Different regulation of SIRT6 on transcriptional and posttranslational level was recently reported by Ronnebaum and colleagues. In this study, the posttranslational regulator of SIRT6 protein was identified and it was shown that the overexpression of ubiquitin ligase CHIP increased SIRT6 protein expression without affecting SIRT6 transcription [28].

Fig. 6 SIRT6 regulates the CSE- and $\text{TNF}\alpha$ -induced MMP1 release. For silencing of SIRT6, synovial fibroblasts from patients with RA ($n=9$) were transfected with control (*ctr*) small interfering RNA (*siRNA*) or with *siRNA* targeting SIRT6 (*S6*) for 48 h and stimulated for additional 24 h with 5 % CSE, 10 ng/ml $\text{TNF}\alpha$ or $\text{TNF}\alpha$ and CSE together. **a** A representative immunoblot confirming successful downregulation of SIRT6 is shown. Molecular weights are indicated in kiloDalton (*kDa*). Vimentin was used as loading control. Reduction of the SIRT6 transcript was confirmed by Real time PCR and is presented as relative expression compared to control transfected cells (*dotted line*). As endogenous control, the expression of HPRT1 mRNA was used for normalization. **b** Protein secretion of MMP1, MMP3, IL8, and IL6 was detected in cell culture supernatants of stimulated and unstimulated (*unst*) RASF by ELISA. **c** Increased protein expression of MMP1 in SIRT6 silenced RASF was confirmed at the mRNA level using Real time PCR analysis and is shown as fold induction compared to untreated cells ($n=7$). As endogenous control the expression of HPRT1 mRNA was used for normalization. Values are mean \pm SEM. n =number of patients investigated. * $p<0.05$; ** $p<0.01$ by Wilcoxon matched-pairs signed rank test



The role of SIRT6 in the regulation of inflammatory mediators has been investigated in various cell types with controversial results. SIRT6 was shown to exhibit pro-inflammatory properties in T cell leukemia and pancreatic cancer cell lines [29, 30]. Consistently, SIRT6 promotes the secretion of $\text{TNF}\alpha$ in mouse embryonic fibroblasts [18]. However, SIRT6 can also attenuate NF- κ B signalling and suppress the expression of NF- κ B-dependent pro-inflammatory genes [24]. In line with this, knockdown of SIRT6 led to increased expression

of pro-inflammatory molecules and pro-angiogenic growth factors in human umbilical vein endothelial cells and primary amnion cells [31, 32]. Moreover, deletion of SIRT6 in mice induced chronic liver inflammation and increased levels of pro-inflammatory cytokines in bone marrow-derived macrophages [33]. Recently, overexpression of SIRT6 was demonstrated to reduce the inflammatory response and tissue destruction in CIA in mice via reduction of $\text{TNF}\alpha$, IL1 β , RANKL, IL6, and IL17 [21]. This study also showed that

overexpression of SIRT6 in RASF followed by treatment with TNF α abolished the expression of MMP1 via inhibition of acetylation on H3K9 at the MMP1 gene promoter. Additionally, the expression of MMP3 was reduced upon overexpression of SIRT6. In our study, we also observed a protective effect of SIRT6 in RASF stimulated with CSE or TNF α , regarding the secretion of MMP1. The expression of MMP3, however, was not affected by SIRT6 knockdown. Different effects of SIRT6 overexpression and knockdown on MMP3 levels indicate that SIRT6 can regulate the expression of MMP3, but the loss of SIRT6 can be compensated by other mechanisms that control MMP3 production. Since MMPs play a key role in the destructive process in RA and are currently not targeted by any therapeutic approach, activators of SIRT6 activity might be an efficient tool to reduce joint destruction.

Due to its different enzymatic activities, SIRT6 can have various functions in the regulation of pro-inflammatory mediators. Whether the different functions of SIRT6 are dependent on the stimulation or the cell type is not known. Therefore, it might be that the functions of SIRT6 in response to inflammation or smoking can vary between cell types as it was shown for the activity of SIRT6 in the regulation of cell survival. Overexpression of SIRT6 selectively induced apoptosis in multiple cancerous human cell lines, but the survival of primary, nontransformed human cells was not affected [34], demonstrating that this function of SIRT6 is cell type specific. Similarly, the silencing of SIRT6 reduced the release of IL8 in a pancreatic cancer cell line, but in our experiments the silencing of SIRT6 did not affect the expression of IL8 in primary RASF [30].

In summary, our study identified SIRT6 as a regulator of cigarette smoke- and TNF α -induced signalling. Stimulation with CSE enhances the pro-inflammatory and matrix-destructive properties of RASF and can further augment the effects of TNF α . At the same time, compensatory mechanisms are initiated that involve the modulation of SIRT6 expression. RASF counteract the effects of the pro-inflammatory stimuli CSE and TNF α by upregulation of SIRT6 thus attenuating the release of the matrix-destructive enzyme MMP1. Therefore, the enhancement of SIRT6 activity could help to reduce the matrix-destructive potential of RASF that is promoted by environmental and inflammatory stimuli and persists despite successful suppression of inflammation using different therapeutic approaches.

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Conflict of interest None.

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